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The risk of recurrence and progression of ductal carcinoma in-situ (DCIS) of the breast is best designated by morphological indicators including nuclear grade and presence of necrosis. To identify molecular alterations underlying these morphological features we have compared gene expression within a cohort of 10 cases of DCIS (6 high-grade or intermediate-grade DCIS with necrosis and 4 low-grade DCIS) using microdissection and cDNA microarray. A set of 42 cDNAs, from a group of 1,500, was identified that were consistently differentially expressed and whose expression profile clustered with DCIS grade. Amongst this set, the angio-associated migratory cell protein (AAMP) was identified as an mRNA that is consistently higher in high-grade DCIS (p= 0.0095) and found to be overexpressed in the T47D breast cancer cell line subjected to hypoxia. Differential expression was confirmed by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and *in situ* hybridization analysis of 37 DCIS. AAMP mRNA tended to be associated with high and intermediate-grade DCIS and DCIS with necrosis. The present study shows that while levels of gene expression are mostly similar between morphologically different DCIS, consistent differences in expression of a subset of genes can be identified between low-grade and high-grade DCIS.

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INTRODUCTION

In recent years there has been an increase in the numbers of ductal carcinoma in-situ (DCIS) and other pre-invasive breast lesions diagnosed (1, 2). As a result, these lesions have become an increasingly significant problem in the evaluation and management of patients with breast disease. To predict the relative risk of recurrence and/or the progression of DCIS to invasive tumors, different classifications have been proposed (3). These are based on a combination of morphological features such as nuclear grade, presence of necrosis, margin width and tumor size and reflect a recognition that DCIS is in fact a spectrum of disease with different morphological characteristics as well as biological and clinical behavior. However, useful as these classifications may be, discordance is common in their use (4) underscoring the need for better predictors of outcome and progression of DCIS.

cDNA microarray analysis is a useful technique for the molecular profiling of the gene expression pattern of cells representing the various stages of malignant transformation (5). Microdissection techniques, including manual and laser capture microdissection (LCM) have recently emerged as effective tools to isolate well defined population of cells from heterogeneous tissue sections as is often encountered in breast cancer (6, 7) Combining microdissection and microarray analysis we investigated the differences between the gene expression patterns of low-grade DCIS and high-grade DCIS in order to identify differentially expressed genes that may be associated with the known different risks of recurrence and progression of these tumor types. It is anticipated that some of the products of the genes identified may serve as molecular

biomarkers for assessing the risk of progression of DCIS or provide targets for new therapies.

Objectives:

The overall aim of the study is to identify genetic molecular markers of the risk of recurrence of in-situ breast lesions. The specific aims are:

Specific aim 1. To identify and clone genes that are differentially expressed between high and low grade DCIS that may contribute to their known risks of recurrence.

Specific aim 2. To study the role of candidate genes identified in specific aim 1 by assessment of expression in-vivo and by manipulations of expression in breast cell lines.

BODY OF REPORT

Accomplishments in the context of the specific aims defined in the statement of work

Specific aim 1.

4 high grade and 2 intermediate grade (all with >10% of ducts containing necrosis), and 4 low-grade human DCIS samples, with homogeneous nuclear grade within each lesion, were obtained from the NCIC-Manitoba Breast Tumor Bank (Department of Pathology, University of Manitoba, Winnipeg, Canada) for microdissection. Tumor samples were microdissected by two methods — a rapid and reliable manual dissection-microscope method previously established in our laboratory and a laser-capture microdissection method using an Arcturus Pixell II instrument (Arcturus Engineering, Inc. Mountain View, CA) depending on the size and the geographical complexity of the DCIS lesions.

^{33}-P-labeled total RNA extracted from microdissected tumor cells were hybridized to the GF200 Human Gene Filters (Research Genetics) containing 5,184 spotted cDNAs. Analysis of our data employing the Pathways 2.01 analysis software (Research Genetics showed that a set of 42 cDNAs, from a group of 1,500 that were expressed well above background levels in our samples, were consistently overexpressed (greater than or equal to 1.8 fold difference in expression) with expression profiles that clustered with DCIS grade. Fourteen transcripts (7 named genes and 7 ESTs) were overexpressed in the low-grade DCIS compared with high-grade/intermediate grade DCIS, whereas 28 transcripts (18 named genes and 10 ESTs) were overexpressed in high-grade/intermediate-grade DCIS compared with the low-grade DCIS lesions. (See Appendix 1 for details and figures)

Specific aim 2

Intraductal necrosis is a distinctive morphological feature of some types of high risk DCIS (8), molecular mechanisms associated with the hypoxia response or activation of the normal response to less severe hypoxia may therefore offer potential indicators of risk of progression in DCIS lesions. To identify genes that were both differentially expressed and that also might be associated with hypoxia, we compared the set of 28 cDNAs consistently overexpressed in high-grade DCIS with 31 cDNAs found to be overexpressed in the T47D cell line subjected to hypoxia, and analyzed in parallel with the DCIS lesions using the same microarray filter. The angio associated migratory cell protein (AAMP) gene was found to be common to both sets of differentially expressed genes and was further assessed by real time quantitative RT-PCR on RNA extracted from the 10 microdissected samples and 28 more DCIS lesions (not microdissected) selected from our Tumor Bank,

hybridization studies on paraffin-embedded sections from these tumors. Differential expression of the mRNA for AAMP was confirmed by both methods, albeit to different significant levels by RT-PCR among the microdissected tumors and the non-microdissected tumors (see details and figures in Appendix 1).

Other genes of interest that are currently being further explored from our list of candidate genes (Tables 2a and 2b, Appendix 1) are: 1. PRCC, the translocation gene in papillary renal cell carcinoma, 2. SLC1A5, the neutral amino acid transporter reported to show elevated levels of expression in multicellular hepatoma spheroids dsplaying central necrosis as seen in high grade DCIS, 3. NDR and SGK, kinases that have been implicated in cell cycle regulation, 4. RALB, a ras related GTP binding protein that showed a high level of differential expression in our pairs of comparisons, 5. HNRNP-F, which is involved in mRNA processing, 6. SDC4, a cell surface protein involved in cell-extracelluar matrix interaction, and 6. CA II. In addition, preliminary work has started on the screening of a human breast cancer expression library, Rapid Screen™ LBC1001(Origene Technologies Inc, Rockville, MD) using primers specific for three of the ESTs overexpressed in high grade DCIS.

KEY RESEARCH ACCOMPLISHMENTS

- Identify, using microdissection techniques and cDNA microarray analysis, a subset of genes consistently differentially expressed between low-grade and high-grade ductal carcinoma in situ (DCIS) of the breast.
- Demonstrate by a combination of cDNA array analysis, real time RT-PCR, and *in-situ* hybridization that expression of the mRNA for angio associated migratory cell protein, previously shown to be associated with angiogenesis and tumor progression (9, 10), is associated with a high grade nuclear morphology and necrosis in DCIS lesions of the breast.

REPORTABLE OUTCOMES

Abstract

Adewale Adeyinka, Ethan D. Emberley, Charles C. Wykoff, Adrian Harris, Leigh C. Murphy and Peter H. Watson. DIFFERENTIAL GENE EXPRESSION ANALYSIS OF MICRODISSECTED DUCTAL CARCINOMA *IN SITU* (DCIS) OF THE BREAST. Proceedings of the American Association for Cancer Research. 42: 58, 2001

Oral presentation

Adewale Adeyinka, Ethan D. Emberley, Leigh C. Murphy and Peter H. Watson. PRE-INVASIVE BREAST CANCERS (DCIS) ASSOCIATED WITH HIGH-RISK AND LOW-RISK OF RECURRENCE DIFFER IN THEIR PATTERNS OF GENE EXPRESSION. Canadian Breast Cancer Research Initiative, Reasons for Hope. May 2001

Manuscript

Adewale Adeyinka, Ethan Emberley, Yulian Niu, Linda Snell, Leigh C. Murphy, Heidi Sowter, Charles Wykoff, Adrian L. Harris, and Peter H. Watson. DIFFERENTIAL GENE EXPRESSION ANALYSIS OF MICRODISSECTED DUCTAL CARCINOMA *IN SITU* (DCIS) OF THE BREAST. (Submitted) Cancer Res

CONCLUSIONS

There are consistent differences in the gene expression patter of high-grade and low-grade in situ breast tumors. Some of these differentially expressed genes may play an important role in determining the biologic and clinical behavior of these tumors and further study is warranted to confirm the differential expression of these genes at the protein level and to determine their relevance to breast cancer progression.

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APPENDICES

Appendix 1

Manuscript

Adewale Adeyinka, Ethan Emberley, Yulian Niu, Linda Snell, Leigh C. Murphy, Heidi Sowter, Charles Wykoff, Adrian L. Harris, and Peter H. Watson. DIFFERENTIAL GENE EXPRESSION ANALYSIS OF MICRODISSECTED DUCTAL CARCINOMA *IN SITU* (DCIS) OF THE BREAST. (Submitted) Cancer Res

Appendix 2

Abstract

Adewale Adeyinka, Ethan D. Emberley, Charles C. Wykoff, Adrian Harris, Leigh C. Murphy and Peter H. Watson. DIFFERENTIAL GENE EXPRESSION ANALYSIS OF MICRODISSECTED DUCTAL CARCINOMA *IN SITU* (DCIS) OF THE BREAST. Proceedings of the American Association for Cancer Research. 42: 58, 2001

Appendix 3

Curriculum vitae

Differential Gene Expression Analysis of Microdissected Ductal Carcinoma *In Situ* (DCIS) of the Breast

Adewale Adeyinka, Ethan Emberley, Yulian Niu, Linda Snell, Leigh C. Murphy, Heidi Sowter, Charles C. Wykoff, Adrian L. Harris, and Peter H. Watson.

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3 The abbreviations used are: AAMP, angio associated migratory cell protein; RT-PCR, reverse transcription-polymerase chain reaction; H&E, Hematoxylin/Eosin; ER, estrogen receptor; PR, progesterone receptor.

Abstract

The risk of recurrence and progression of ductal carcinoma in-situ (DCIS) of the breast is best designated by morphological indicators including nuclear grade and presence of necrosis. To identify molecular alterations underlying these morphological features we have compared gene expression within a cohort of 10 cases of DCIS (6 high-grade or intermediate-grade DCIS with necrosis and 4 low-grade DCIS) using microdissection and cDNA microarray. A set of 42 cDNAs, from a group of 1,500, was identified that were consistently differentially expressed and whose expression profile clustered with DCIS grade. Amongst this set, the angio-associated migratory cell protein (AAMP) was identified as an mRNA that is consistently higher in high-grade DCIS ($p= 0.0095$) and found to be overexpressed in the T47D breast cancer cell line subjected to hypoxia. Differential expression was confirmed by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) and *in situ* hybridization analysis of 37 DCIS. AAMP mRNA tended to be associated with high and intermediate-grade DCIS and DCIS with necrosis. The present study shows that while levels of gene expression are mostly similar between morphologically different DCIS, consistent differences in expression of a subset of genes can be identified between low-grade and high-grade DCIS.

Introduction

In recent years there has been an increase in the numbers of ductal carcinoma in-situ (DCIS) and other pre-invasive breast lesions diagnosed (1, 2). As a result, these lesions have become an increasingly significant problem in the evaluation and management of patients with breast disease. To predict the relative risk of recurrence and/or the progression of DCIS to invasive tumors, different classifications have been proposed (3). These are based on a combination of morphological features such as nuclear grade, presence of necrosis, margin width and tumor size and reflect a recognition that DCIS is in fact a spectrum of disease with different morphological characteristics as well as biological and clinical behavior. However, useful as these classifications

may be, discordance is common in their use (4) underscoring the need for better predictors of outcome and progression of DCIS.

As already noted, intraductal necrosis is a distinctive morphological feature of some types of high risk DCIS (5) and is believed to be attributable to the presence of severe hypoxia that can arise within the duct through an imbalance between metabolic requirements and blood supply (5). Thus although comparatively little is known about the genetic and molecular events that are responsible for the progression of some DCIS to invasive breast carcinoma, alterations in the molecular mechanisms associated with the hypoxia response or activation of the normal response to less severe hypoxia may therefore offer potential indicators of risk of progression in DCIS lesions.

cDNA microarray analysis is a useful technique for the molecular profiling of the gene expression pattern of cells representing the various stages of malignant transformation (6). Microdissection techniques, including manual and laser capture microdissection (LCM) have recently emerged as effective tools to isolate well defined population of cells from heterogeneous tissue sections as is often encountered in breast cancer (7, 8) Combining microdissection and microarray analysis we investigated the differences between the gene expression patterns of low-grade DCIS and high-grade DCIS in order to identify differentially expressed genes that may be associated with the known different risks of recurrence and progression of these tumor types. It is anticipated that some of the products of the genes identified may serve as molecular biomarkers for assessing the risk of progression of DCIS or provide targets for new therapies.

Materials and Methods

Human breast tumor samples

Human DCIS samples were obtained from the NCIC-Manitoba Breast Tumor Bank (Department of Pathology, University of Manitoba, Winnipeg, Canada) (9). All cases in the bank have been rapidly frozen at 70 ^{0}C after surgical removal and subsequently processed to create formalin-fixed paraffin-embedded tissue blocks and

matched frozen tissue blocks with mirror image surfaces corresponding to the formalin-fixed tissue blocks. Histological interpretation and assessment of every sample in the Bank is done on hematoxylin/eosin (H&E)-stained sections from the paraffin tissue by a pathologist.

Two cohorts of tumors were selected. The first cohort of 10 tumors comprised DCIS lesions with homogenous nuclear grade within each lesion. These included 4 high grade and 2 intermediate grade (all with >10% of ducts containing necrosis), and 4 low-grade DCIS cases and were used as the primary microdissection series. The second cohort comprised 28 DCIS specimens used to confirm the differential expression observed in the microdissected cases. Tumor classification and the evaluation of intraductal necrosis were done on high quality hematoxylin and eosin stained slides derived from the formalin fixed and paraffin embedded blocks and agreed on by 2 pathologists (AA and PHW), independent of the array or subsequent gene expression analysis. The classification of DCIS into histological grades was done according to the Van Nuys grading system (10) and tumors were assigned to the highest histological grade present in the tissue section studied. Both cohorts combined (38 tumors) included 17 non-high grade DCIS (7 with necrosis and 10 without necrosis) and 21 high-grade DCIS. Twenty-seven tumors were estrogen receptor (ER)-positive, 11 were ER-negative, 25 were progesterone receptor (PR)-positive and 13 were PR-negative (Table 1). Steroid receptor status was determined by ligand-binding assay. A positive ER status and positive PR status were defined as more than 3 fmol/mg protein and more than 15 fmol/mg protein, respectively.

Microdissection

Tumor samples were microdissected by two methods, depending on the size and the geographical complexity of the DCIS lesions. A manual dissection-microscope method previously established in our laboratory (7) was used where possible as it is rapid and reliable, however a laser-capture microdissection method using an Arcturus Pixell II instrument (Arcturus Engineering, Inc. Mountain View, CA) was used for two cases with a heavy inflammatory cell infiltrate of the stroma around the ducts and to obtain epithelial cells from a normal breast

sample. For the manual microdissection, tumor cells were dissected from 6-10 20-μm frozen sections of tumors mounted on agarose gel dropped onto a plain glass slide and stained briefly with H&E, as previously described (7). For laser microdissection, tumor cells were microdissected from 14-17 10-μm frozen sections mounted onto plain glass slides and stained with H&E according to the Arcturus Engineering protocol (http://www.arctur.com/technology/protocols.html).

Tissue RNA extraction

Total RNA was extracted with Trizol Reagent (Life Technologies, Inc.) from tumor cells obtained from all 10 microdissected DCIS using a small scale RNA extraction protocol (7). Total RNA was similarly extracted from six 20-μm frozen sections from each of the 28 DCIS tumors constituting the second cohort.

Cell line culture and RNA extraction

The T47D human breast cancer cell line was obtained from the Imperial Cancer Research Fund (ICRF) cell service, and grown in DMEM , RPMI or Hams F-12 supplemented with 10% fetal calf serum (Globepharm), L-glutamine (2 μM), penicillin (50 IU/ml) and streptomycin sulphate (50 μg/ml). Parallel incubations were performed on flasks of cells approaching confluence in normoxia (humidified air with 5% CO_2) or hypoxia. Hypoxic conditions were generated in a Napco 7001 incubator (Precision Scientific) with 0.1% O_2, 5% CO_2 and balance N_2. Total RNA was prepared according to Chomczynski and Sacchi (11) and the quality assessed by absorbance at 260/280nm as well as by electrophoresis in 1% agarose gels by staining of the 28S rRNA with ethidium bromide.

Microarray cDNA membranes Human GF-200 cDNA Microarray membranes and the Pathways 2.01 analysis software were purchased from Research Genetics, Inc. Reverse transcription, ^{33}P-labeling and hybridization of RNA to array membranes were done according manufacturer's instructions. Briefly, for each sample, 1μg of total RNA was reverse transcribed in the presence of 10 μl [^{33}P]dCTP at a concentration of 10 mCi/ml, dATP, dGTP,

dTTP at 20 mM, 500ng of Oligo-dT and 200 units of SuperScript II RT (Life Technologies, Inc.), all in a 30 μl volume. The labeled cDNA was purified by passing through a Bio-Spin 6 Chromatography column (Bio-Rad), denatured and hybridized to human GF-200 cDNA microarray membranes. Membranes were pre-hybridized at 42 ^{0}C for at least 2 hours in 5 ml microhyb solution (Research Genetics, Inc) in the presence of 1.0 μg/ml poly-dA and 1.0 μg/ml Cot 1 DNA. After an overnight (20hr) hybridization with labeled cDNA, membranes were washed, exposed to Imaging screen-K (Bio-Rad) and scanned in a phosphorimager (Bio-Rad) after which they were stripped and reused three times. The tiff images (Figure 2) obtained from the phosphorimager were imported into the Pathways 2.01 analysis software (Research Genetics, Inc.) for analysis and comparison between different membranes from different tumors. Membranes to which ^{33}P-labeled reverse transcribed RNA from high-grade or non-high grade DCIS with necrosis was hybridized were compared with membranes to which ^{33}P-labeled reverse transcribed RNA from low-grade DCIS (non-high grade DCIS without necrosis) was hybridized. To compare two membranes, an all-data-point method of normalization was used and cDNA showing expression levels lower than 10X background were masked from comparison. A 1.8 fold or greater differential expression was used as our cut off level for overexpression of any particular transcript. Each cDNA spot on the Pathways pseudo-color membrane showing >= 1.8 fold differential expression was examined by direct vizualization to eliminate those that might be false positives — spots judged to be not properly centered or spots influenced by 'bleed-over' from adjacent spots. A similar approach was used to compare profiles of gene expression between normoxic and hypoxic T47D breast cells.

Real time quantitative RT-PCR

Total RNA from the 10 microdissected samples used for the GeneFilter hybridization and the 28 DCIS samples of the second cohort were reverse transcribed in a total volume of 20 μl as described previously (7). For each sample, 2 μl of 0.1 μg/μl of total RNA was added to an 18 μl RT mix (4 μl of 5X RT buffer; 1 μl each of dATP, dCTP, dGTP, and dTTP, all at a concentration of 2.5 mM; 2 μl of 0.1% bovine serum albumin (BSA); 2 μl of 100

mM dithiothreitol (DTT); 2 μl of dimethyl sulfoxide (DMSO); 2 μl of 50 μM Oligo-dT primer, and 2 μl of 200 units/μl of Moloney murine leukemia virus reverse transcriptase) and incubated at 37°C for 1.5 hrs. The resulting cDNA was diluted with 20 μl of sterile water and used as template for the quantitative RT-PCR.

The mRNA sequences of the genes identified using the array membranes and corresponding Research Genetics information were determined by using the blast module of National Center for Biotechnology Information database. Primers that specifically detect these sequences were designed and employed for the RT-PCR reaction using the LightCycler Instrument (Roche Molecular Biochemicals) and the LightCycler-DNA Master SYBR Green I reaction mix for PCR (Roche Molecular Biochemicals), containing the SYBR Green I dye as detection format. For each sample, triplicate reactions were set up in capillaries with the following reaction mix: 0.33 μl DNA template; 0.2 μl each of 50 mM sense and antisense primers; 1.6 μl of 25 mM $MgCl_2$; 2 μl LightCycler-DNA Master SYBR Green I reaction mix, and 16 μl sterile water. For each batch of reactions controls included RT-negative and RNA-negative controls, and serial dilutions (1 ng, 0.01 ng and 0.0001 ng) of plasmid DNA as standards for linear regression analysis of unknown samples. The denaturation, amplification, melting curve analysis, and cooling programs of the LightCycler instrument were set according to manufacturer's specifications. The annealing temperature and elongation time, however, were set depending on primers and product length, respectively. PCR products were run on a 1.5% agarose gel to confirm the PCR specificity. The expression of Cyclophilin 33A, a house keeping gene, was used to normalize for variances in RNA and cDNA input. The AAMP primers were sense, 5'–CGC CTG CTT ACT GAC TAC C–3' and antisense, 5'-GTA TCT CTT CCT CCT TTC CAC-3', with annealing temperature of 57 °C and elongation time of 20 seconds. The cyclophilin 33A primers were sense, 5'-GCT GCG TTC ATT CCT TTT G-3' and antisense 5'-CTC CTG GGT CTC TGC TTT G -3', with annealing temperature of 60 °C and elongation time of 10 seconds.

In situ hybridization

Paraffin embedded 5 μm breast tumor sections were analyzed for AAMP mRNA expression by in situ hybridization, according to a previously described protocol (12). The plasmid pT7T3D-pac containing a 460-bp insert of the human

AAMP cDNA (IMAGE consortium clone Id 789011, GenBank accession no. AA452988) was used as a template to generate sense and antisense probes. UTP ^{35}S-labeled riboprobes were synthesized using Riboprobe Systems (Promega, Madison, WI) according to manufacturer's instructions. Sense probes were used as controls. In situ hybridization and washing conditions were as described previously (12). Sections were developed, after 5 weeks, using Kodak NTB-2 photographic emulsion and counterstained with Lee's stain (12).

Levels of AAMP mRNA expression were assessed in the sections by microscopic examination using a semiquantitative approach (12). Scores were obtained by estimating the average signal (on a scale of 0-3) and the proportion of ductal cells showing a positive signal (0, none; 0.1, less than 10%; 0.5, less than 50%; 1.0, greater than 50%). The intensity and proportion score were then multiplied to give an overall score.

Statistics

The Mann-Whitney U test, the Fisher's exact test, and Spearman's correlation coefficient were used as appropriate. To determine the reproducibility of hybridization results with the GF-200 membrane, the normalized intensity values of all the 5,568 gene spots from duplicate hybridizations of duplicate ^{33}P-labeled cDNA of the same RNA sample from the hypoxic T47D cells — one hybridization was to a fresh membrane and the other to a stripped membrane — were compared by linear regression and correlation analyses. For all tests, statistical significance was considered to be at the $p < 0.05$ level (Graphpad prism, Graphpad Software, San Diego, CA.).

Results

Microdissection and microarray cDNA filters

The manual and laser assisted microdissection techniques proved to be equally effective in isolating ductal carcinoma cells from our DCIS specimens (Figure 1). We were able to isolate approximately 2 μg of total RNA from 14-17 10-μm frozen sections of the two tumors and the normal sample subjected to the laser assisted method of dissection, whereas the manual dissection method yielded approximately 2-4 μg of total RNA from 6-10 20-μm frozen sections of tumor samples. The quality of hybridization signals produced by the labeled reverse transcribed total RNA obtained by both dissection techniques were also comparable as assessed by the exposure time needed to obtain equivalent signal intensities for analysis.

For the purpose of analysis, the six DCIS tumors with high or intermediate grade nuclear morphology and necrosis were grouped together and signals from each hybridized cDNA microarray membrane was compared with that for each of the four low-grade tumors (Figure 2A-C) resulting in 24 pairs of comparisons. After masking those transcripts showing expression levels lower than 10X background, we were left with approximately 1,500 cDNAs for our comparison analysis. The level of consistency of the array hybridization was examined in duplicate hybridizations of duplicate ^{33}P-labeled cDNA of the same RNA sample from the T47D cell line (one hybridization was to a fresh membrane and the other to a stripped membrane) to the GF-200 membrane, which demonstrated that a high level of consistency could be obtained (r^2 =0.88, data not shown). Nevertheless, to exclude the transcripts that might be differentially expressed due to individual differences between the tumors or due to variations in hybridization conditions between each experiment, we have included in our list of differentially expressed transcripts only those that were differentially expressed in at least 9 pairs of comparison (Table 2). This ensures only the inclusion of transcripts that were differentially expressed in at least three different high-grade/intermediate-grade DCIS compared with the low-grade DCIS samples. Using this selection criterion, 14 transcripts (7 named genes and 7 ESTs) were overexpressed in the low-grade DCIS compared

with high-grade/intermediate grade DCIS, whereas 28 transcripts (18 named genes and 10 ESTs) were overexpressed in high-grade/intermediate-grade DCIS compared with the low-grade DCIS lesions.

To assess the relationship between patterns of gene expression and DCIS histology, a two-way average linkage cluster analysis was applied (with the Cluster program) (13) to organize the 42 consistently differentially expressed genes and tumors such that genes and tumors with similar expression patterns are clustered together and adjacent to one another (13). Normalized gene expression data were logarithmically transformed and a pairwise average linkage hierarchical clustering was performed using an uncentered correlation for both array and gene-clustering dimensions. The resulting phylogenetic tree (visualized with the Treeview program) (13) showed that, apart from one high-grade tumor, all the high- and intermediate-grade DCIS were closely related to each, separating on the same major limb of the tree. Two of the four low-grade tumors also clustered to this arm of the tree but they separated further down the arm along with two other higher-grade tumors from which they finally separated. The two other low-grade tumor were more related to a normal breast sample than they were to five of the six higher-grade tumors (Figure 2D). To identify genes that were both differentially expressed and that also might be associated with hypoxia, we compared the set of 28 cDNAs consistently overexpressed in high grade DCIS with 31 cDNAs found to be overexpressed in the T47D cell line subjected to hypoxia, and analyzed in parallel with the DCIS lesions using the same microarray filter. The AAMP gene was found to be common to both sets of differentially expressed genes and was assessed further. The mRNA for AAMP was differentially overexpressed in 11 pairs of tumor comparisons (mean 2.1 fold across two high-grade DCIS and one intermediate-grade DCIS compared with the low-grade DCIS lesions). AAMP was similarly overexpressed in T47D cells analyzed by array (mean 2.5 fold) subjected to hypoxic as compared to normoxic conditions.

Real time quantitative RT-PCR for AAMP mRNA

AAMP mRNA expression was then assessed by RT-PCR in the 10 original microdissected tumors and subsequently in 28 additional non-microdissected DCIS samples. RT-PCR assay in one sample from the second DCIS cohort failed to yield a product with the control. AAMP expression in the microdissected ductal epithelium from the initial 10 DCIS

tumors was higher in high- and intermediate-grade DCIS (P=0.0095, Mann Whitney) compared with the low-grade DCIS (Figure 3A). Among the 27 non-microdissected DCIS series, total AAMP expression within the tumor section tended to be associated with higher grade and necrosis. (P=0.0427, one-tailed, Mann Whitney) (Figure 3B). The overall expression level of AAMP in these 27 tumors did not show any association with either the progesterone receptor (p=0.55, Mann Whitney) or estrogen receptor (p=0.37, Mann Whitney) status of the tumors.

In situ hybridization (ISH)

AAMP mRNA expression was also assessed by *in situ* hybridization in all 37 tumors. The AAMP anti-sense probe showed stronger signals for AAMP mRNA in high-grade DCIS ducts and DCIS ducts with necrosis as compared with low-grade ducts, with variation from duct to duct in individual tumors. However there was no marked gradation in expression from luminal to stromal aspect of ducts with central necrosis. Expression was also observed in blood vessels in the stroma around the ducts (Figure 4). The *in situ* hybridization intensity score assigned to each tumor was based on an average of the epithelial expression assessed from all the ducts in the sections examined. AAMP expression detected by ISH showed some relationship to grade and necrosis, though this association was at best of borderline significance. AAMP ISH scores were higher in high/intermediate/high grade DCIS (p=0.05, Fisher's exact test) and in tumors with necrosis (p=0.09, Fisher's exact test) compared with low grade and those without necrosis, respectively. There was no association between the levels of AAMP detected by ISH and the estrogen receptor (p=0.2, Fisher's exact test) or progesterone receptor (p= 0.5, Fisher's exact test) status of tumors.

Discussion

We have shown that while levels of gene expression are mostly similar between morphologically different DCIS, consistent differences in expression of a subset of genes can be identified between low-grade and high-grade DCIS. Amongst these differentially expressed genes the angio-associated-migratory cell protein (AAMP) was found to be

expressed at a higher level by most high-grade DCIS and DCIS with necrosis and is also overexpressed in a breast cancer cell line subjected to hypoxia.

The two microdissection techniques employed in the present study were effective in isolating ductal cells from our samples. The laser-assisted microdissection, however, limits the thickness of sections for dissection to a maximum of 10 μm hence the need to dissect more sections in order to obtain a reasonable yield of RNA for hybridization. An alternate approach would be to dissect a minimum number of cells to obtain enough RNA that could serve as a template for transcriptional-based RNA amplification (8). Our approach of dissecting cells from several sections, though seemingly burdensome, avoids the technical difficulties associated with RNA amplification.

cDNA microarray analysis is a powerful tool for the simultaneous analysis of large sets of genes and for the profiling of different tissue types. Studies have, however, shown that any single microarray output is subject to some variability and that pooling of data from replicates can provide a more reliable classification of gene expression (14). In the present study, the small amount of RNA available from some of our microdissected samples precluded us from carrying out replicate array experiments on the same sample. We have, however, attempted to address the issue of variability by pooling data from multiple comparisons of different tumors and selecting only those transcripts that are consistently differentially expressed in our comparisons. This we believe may be more stringent than replicate experiments on the same sample.

Aside from the present study, there exists no gene expression profiling data comparing different grades of DCIS. However, similar to our findings, loss of heterozygosity (LOH)/allelic imbalance studies as well as comparative genomic hybridization (CGH) studies have shown that there are distinct genetic changes associated with the level of differentiation/grade and morphology of DCIS of the breast (15, 16, 17). Nevertheless, our present approach of using gene expression profiling has the advantage of identifying specific genes that may be important in the differentiation and progression of DCIS, whereas LOH and CGH studies can only identify larger genomic areas harboring several genes.

The hierarchical cluster algorithm operating on a very small set of samples (10 tumors and 1 normal breast sample) and expression data (42 genes and ESTs), was, apart from one high-grade tumor, able to group the higher grade DCIS

lesions together (Figure 2D). Contrary to what might have been expected, that is, a clear-cut demarcation between the low-grade and higher-grade tumors based on the selected subset of differentially expressed genes, two low-grade tumors clustered on the same limb of the dendogram as the higher grade tumors. These two low-grade tumors, even though they still maintained some close relationship to two of the higher-grade tumors, eventually separated from the higher-grade tumors lower down the limb of the tree. This is because gene expression analysis, aside from being able to reproduce, to a reasonable extent, the classification of neoplastic lesions based on morphological parameters is also able to reveal subtle molecular relationships between lesions in different morphological groups. The genes and ESTs more highly expressed in the low grade-tumors all clustered together (cluster 1, figure 2D) and showed expression in the normal tissue sample, indicating that these transcripts were probably downregulated in the higher grade tumors showing low levels of expression. On the other hand, the transcripts showing a high level of expression in the higher grade tumors (clusters 2 and 3, figure 2D) all showed low levels of expression in the normal tissue as well as in the low-grade tumors (especially cluster 2), an indication that these transcripts were upregulated in these higher grade tumors.

Of interest among the cluster 1 genes are syndecan 4 (*SDC4*), metalloproteinase inhibitor 1, and *N-RAS*. In addition to the antiangiogenic function of metalloproteinase inhibitor 1, it shares with *SDC4* a role in cell-extracellular matrix interaction to inhibit invasion (18). *N-RAS*, though able to induce anchorage independent growth, is unable to induce an invasive phenotype in breast cancer cells (19). In addition, matrix metalloproteinases are known to play an important role in RAS-mediated invasiveness of human epithelial cells (19).

Cluster 2 transcripts were of low expression in the low grade-tumors, whereas cluster 3 transcripts showed a moderate level of expression in the two low-grade tumors on the same limb of the phylogenetic tree as the higher grade tumors. Among the cluster 2 (figure 2D) genes, the kinases, *NDR* and *SGK*, and the phosphatase *PPP6C*, are serine/threonine specific in their catalytic activities and they have been implicated in cell cycle regulation (20, 21, 22). In addition, *NDR* and *PPP6C* regulate cell morphology (21, 22) whereas SGK is able to prevent the fork head transcription factor (FKHRL1) induced apoptosis (23) *UBE2I*, the human homolog of the yeast *UBC9* that mediates the transition from G1 to S-phase of the cell cycle (24), is known to interact with *RAD51* and *RAD52*, which are part of the *BRCA1* pathway

(25, 26) as well as with *TP53* (25). *PRCC*, the translocation gene in papillary renal cell carcinoma is speculated to function in the signaling cascade because it possesses a proline-rich domain (27). Its recent association with graft-versus-host reaction (28) raises the possibility that it might play a role in inflammatory response that is frequently associated with high-grade comedo type DCIS. Up-regulation of desmocollin 2 (an intracellular desmosome junction protein) mRNA in some of the high-grade DCIS leaves room for one to speculate that loss of this gene may be important for the transition of DCIS to invasive tumor since a member of the desmocollin subfamily of the cadherin superfamily, desmocollin 3, has been reported to be downregulated in invasive breast cancer (29)

Carbonic anhydrase II (*CA II*), a cytosolic carbonic anhydrase (CA) isoenzyme is highly expressed in several tumor types including gastric, colorectal, and pancreatic carcinomas (30, 31, 32) as well as malignant brain tumors (33) and its expression has been shown to correlate with biological aggressiveness of rectal cancer (32). Furthermore we have recently found that altered expression of other carbonic anhydrases, *CA IX and CAXII*, is a frequent occurrence between low- and high-grade DCIS (34). The expression of these transmembrane isoenzymes is influenced by hypoxia and differentiation and they are expressed on different aspects of the breast cell membrane where they may act to influence the local extracellular pH surrounding the cancer cells thereby possibly creating a microenvironment conducive for tumor growth and spread (35) In preliminary experiments we have not be able to demonstrate hypoxia regulation of *CA II* in breast cell lines (data not shown) and a similar role for the intracellular *CA II* in the progression of breast cancer remains to be determined. However, higher levels of the intracellular *CA II* in high grade DCIS may be part of coordinated changes in pH regulation, and by causing an increased generation of intracellular CO_2, *CA II* may facilitate the actions of the extracellular CA isoenzymes (36)

The cluster 3 gene (figure 2D), human neutral amino acid transporter B (*SLC1A5*) was reported to show elevated levels of expression in multicellular hepatoma spheroids displaying central necrosis, similar to that seen in high grade DCIS, when compared with single cell suspension. This elevated level of *SLC1A5* mRNA paralleled changes in glutamine uptake by tumor cells in this model, suggesting that a hypoxic tumor microenvironment impacts on the uptake of specific nutrients (37). Since one important goal of expression profiling is to develop a molecular based classification

system for tumors (38, 39), further expression-profiling studies of a larger series of DCIS of the breast would be necessary to give insight into the importance of these genes in developing a molecular signature for DCIS of the breast.

AAMP was first isolated from a human melanoma cell line as a motility-associated cell protein and was found to be expressed strongly in endothelial cells, cytotrophoblast, and poorly differentiated colon adenocarcinoma cells found in lymphatics (40). AAMP has two immunoglobulin domains and six WD40 repeat domains, suggesting possible membership in both the immunoglobulin superfamily and the WD40 repeat family of proteins (40, 41). The presence of both the immunoglobulin type domains and WD40 repeats sequence motifs in AAMP implies a multifunctional role for this protein (41). Experimental evidence to date suggests that AAMP may play a role in cell motility and angiogenesis. This has been examined and demonstrated in endothelial cells (42). However, we and others have shown that AAMP is expressed by other cells and this property, which may be related to the fact that it shares a common epitope with α-actinin and a fast twitch skeletal muscle fiber protein (41), is not necessarily restricted to endothelial cells and angiogenesis (43). However, this extra-epithelial source of AAMP would probably account for the inability to fully reproduce the AAMP-grade/necrosis association demonstrated in our microdissected series in the non-microdissected cohort, underscoring the usefulness of microdissection techniques in profiling ubiquitously expressed genes from specific cell types in a heterogeneous tissue environment.

Necrosis is believed to represent the extreme manifestation of hypoxia in tissues (44). The finding that AAMP is induced in-vitro in a breast cell line subjected to hypoxia and also in-vivo in DCIS associated with necrosis, suggest that AAMP may be a hypoxia-regulated gene that may influence growth and survival of DCIS. However, necrosis may be attributable to other causes and alternative stresses may influence gene expression in cells subjected to hypoxia (45). Regulation by hypoxia in-vitro may also not be the only or dominant factor in the complex in-vivo environment (34). Further work will be required to establish if AAMP expression is directly regulated by mediators of hypoxia response and to confirm the observation here that expression in-vivo may be indicative of hypoxia.

In summary, we have shown that high grade DCIS can be distinguished from low grade DCIS by the pattern of gene expression and that upregulation of AAMP, a gene that has previously been associated with angiogenesis and tumor progression, is also associated with a high nuclear grade morphology and necrosis in DCIS of the breast.

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Table 1. Morphology, AAMP expression, and steroid receptor status of 37 DCIS of the beast

Serial #[1]	LAB#	Nuclear grade	Necrosis	AAMP RT-PCR	ER status[2]	PR status[3]
1	12024	HG	+	0.21	-	-
2	10046	HG	+	0.13	-	-
3	13110	HG	+	0.34	+	-
4	13049	HG	+	0.31	+	+
5	11970	IG	+	0.31	-	-
6	11722	IG	+	0.11	+	+
7	10047	LG	-	0.10	+	+
8	11161	LG	-	0.09	+	+
9	9062	LG	-	0.10	+	+
10	10919	LG	-	0.11	+	+
11	12340	HG	+	1.30	-	-
12	13115	HG	+	0.63	-	+
13	15200	HG	+	1.03	+	-
14	11972	HG	+	0.53	-	-
15	12054	HG	+	0.56	-	-
16	12485	HG	+	0.39	+	-
17	15242	HG	+	0.37	-	+
18	11202	HG	+	0.72	+	+
19	12354	HG	+	0.34	+	+
20	13523	HG	+	0.39	+	+
21	13423	HG	+	0.34	+	-
22	12338	HG	+	1.08	+	+
23	14815	HG	+	0.58	+	+
24	15134	HG	+	0.49	+	+
25	13005	HG	+	0.46	-	-
26	11442	HG	-	1.33	+	+
27	15344	IG	+	0.55	+	+
28	11285	IG	+	0.72	+	-
29	14902	IG	+	0.74	-	+
30	15108	IG	+	0.46	+	+
31	15010	IG	+	1.22	+	+

Table 1 continued

Serial #[1]	LAB#	Nuclear grade	Necrosis	AAMP RT-PCR	ER status[2]	PR status[3]
32	10351	IG	-	0.38	+	+
33	15439	IG	-	0.32	+	+
34	12438	IG	-	0.64	-	-
35	12571	IG	-	0.44	+	+
36	13686	LG	-	0.44	+	+
37	15284	LG	-	0.47	+	+

Table 2a. Classification of genes consistently upregulated in high/intermediate-grade DCIS compared with low-grade DCIS of the breast

Accession Number	Gene name	Gene symbol	Average fold change	S.D.	Fold change range	Number of comparisons[1]
Cell Adhesion/Cell motility						
AA428778	Human T cell leukemia LERK-2 (EPLG2)	*EFNB1*	6.9	5.3	2.1-10.0	12
AA452988	Homo sapiens angio-associated migratory cell protein (AAMP)	*AAMP*	2.1	0.2	1.8-2.4	11
AA074677	Human desmocollin-2	*DSC3*	2.1	0.2	1.9-2.4	10
Cell cycle/Cell proliferation regulator						
H73724	Cyclin-dependent kinase 6	*CDK6*	4.4	2.7	2.1-10.3	12
R25074	Human transmembrane 4 superfamily protein (SAS)	*SAS*	8.1	7.1	2.3-14.0	12
Kinase/Phosphatase						
AA521346	Ndr protein kinase	*NDR*	2.6	0.6	2.0-4.0	13
AA486082	Putative serine/threonine protein kinase	*SGK*	3	0.7	2.0-3.6	12
AA521083	Protein phosphatase 6	*PPP6C*	2.6	0.4	2.1-3.2	12
Transcription factor						
AA488233	H.sapiens mRNA for prcc protein	*PRCC*	2.9	0.4	2.2-3.5	12
AA478436	Human SWI/SNF complex 60 KDa subunit (BAF60b)	*SMARCD2*	2.2	0.3	1.9-2.6	9
Receptors and signal transduction						
W39343	V-ral simian leukemia viral oncogene homolog B (ras related; GTP binding protein)	*RALB*	9.5	2.2	6.5-13.5	12
AA598508	Cellular retinoic acid-binding protein 2	*CRABP2*	2.1	0.3	1.8-2.6	9

Table 2a continued

Accession Number	Gene name	Gene symbol	Average fold change	S.D.	Fold change range	Number of comparisons[1]
Cellular Metabolism						
H23187	Carbonic anhydrase II	*CA2*	2.3	0.2	2.0-2.7	9
AA487197	Ubiquitin-conjugating enzyme E2I (homologous to yeast UBC9)	*UBE2I*	2.3	0.4	1.8-2.9	9
AA070997	Proteasome (prosome, macropain) subunit, beta type, 6	*PSMB6*	2.1	0.2	1.8-2.5	9
Molecule Transporter						
T70098	Human neutral amino acid transporter B	*SLC1A5*	6.6	5.9	1.9-10.6	12
RNA binding and RNA processing						
AA490991	Homo sapiens HnRNP F	*HNRNPF*	2.1	0.2	1.8-2.6	11
HUGE						
H75699	KIAA0297 gene	*KIAA0297*	7	5	2.4-17.8	9

Table 2b. Classification of genes consistently upregulated in low-grade DCIS compared with high/intermediate-grade DCIS of the breast

Accession number	Gene name	Gene symbol	Average fold change	S.D.	Fold change range	Number of comparisons[1]
DNA binding						
N54596	Human Krueppel-related zinc finger protein (H-plk)	*H-PLK*	2.4	0.4	1.9-2.9	12
AA452725	Nucleobindin precursor	*NUCB1*	2	0.2	1.8-2.4	9
Cell surface protein						
AA148736	Syndecan 4 (amphiglycan, ryudocan)	*SDC4*	2.4	0.5	1.8-3.2	11
Translation regulation						
R86304	Eukaryotic translation initiation factor 2B	*EIF2B2*	1.9	0.1	1.8-2.1	9
Cell cycle/Cell proliferation regulator						
AA504682	Neuroblastoma RAS viral (v-ras) oncogene homolog	*NRAS*	2.2	0.3	1.8-2.8	12
R82299	S-adenosylmethionine decarboxylase 1	*AMD1*	2.2	0.2	1.9-2.7	11
Transcription factor						
AA504682	SNF2 (sucrose nonfermenting, yeast, homolog)-like 1	*SMARCA1*	2.3	0.4	1.8-3.0	12

Table & Figure Legends

Table 1

[1]serial # 1-10 = microdissected tumors

[2]ER less than 3.0 fmol/mg protein = -; ER more than 3.0 fmol/mg protein = +ve

[3]PR less than 15.0 fmol/mg protein = - ; PR more than 15.0 fmol/mg protein = +ve

Table 2

[1] Numbers of comparisons (maximum number 24) between pairs of DCIS that show differential expression for a particular gene (i.e., comparisons between each of 6 intermediate/high-grade DCIS with each of 4 low-grade DCIS).

Figure 1. Laser-capture microdissection of duct cells from H&E stained frozen section (10 μm) of a high-grade DCIS of the breast. (A) Three ducts (1, 2 and 3) identified for dissection (B) Ducts after dissection (C) Cells from duct successfully captured and transferred.

Figure 2. A-C, Differential gene expression analysis employing the GF200 cDNA array membrane and the Pathways 2.01 analysis software. Hybridization of ^{33}P-labeled reverse transcribed RNA from a high grade DCIS (A) and a low grade DCIS (B) to Human GF200 cDNA array membranes. (C) Portion of synfilter generated by the Pathways 2.01 analysis software from membranes A and B. Yellow spots show genes expressed equally by both samples, green spots; genes differentially expressed by high grade tumor and red spots; genes differentially expressed by low grade tumor. AAMP is represented by green spot sorrounded by blue rectangle. D, Cluster map and phylogenetic tree resulting from an average-linkage cluster analysis of the 42 differentially expressed genes identified in our series of DCIS. Each color patch represents the expression level of the associated gene in that tissue sample with a continuum of expression from bright green (lowest) to bright red (highest). N50 is a normal breast sample, HG, high-grade DCIS; LG, low-grade DCIS; IG, intermediate grade DCIS; 1, cluster of genes and ESTs differentially expressed in low-grade DCIS; 2 and 3, cluster of genes and ESTs differentially expressed in intermediate/high-grade DCIS.

Figure 3 A, AAMP mRNA expression detected by RT-PCR shown relative to grade and necrosis in 10 microdissected DCIS samples. (necrosis +ve, high-grade DCIS or non-high grade DCIS with necrosis. n=6; necrosis –ve, non-high-grade DCIS without necrosis. n=4. B, AAMP mRNA expression detected by RT-PCR shown

relative to grade and necrosis in 27 DCIS of the breast. (necrosis +ve, high-grade DCIS or non-high grade DCIS with necrosis. n=21; necrosis –ve, non-high-grade DCIS without necrosis. n=6.

Figure 4. In situ hybridization with ^{35}S-labeled anti-sense probe for AAMP showing strong signals for AAMP in high-grade ducts with necrosis (A and B) and weaker signals in low-grade DCIS (D). There were no signals with the sense probe (C), x 40.

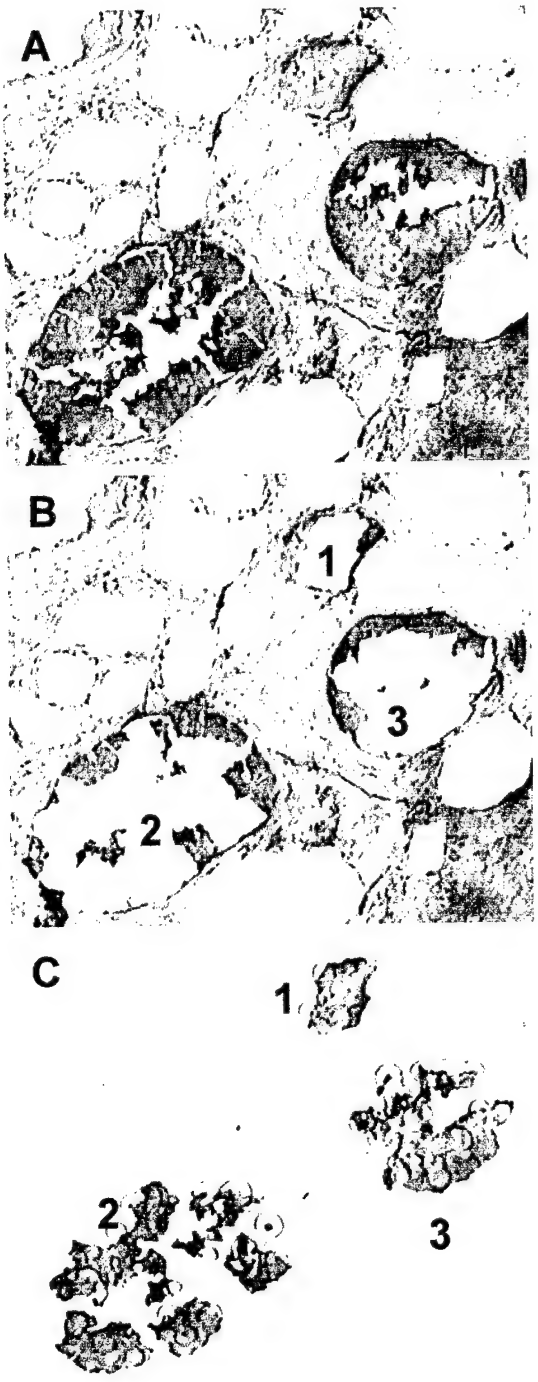


Figure 1

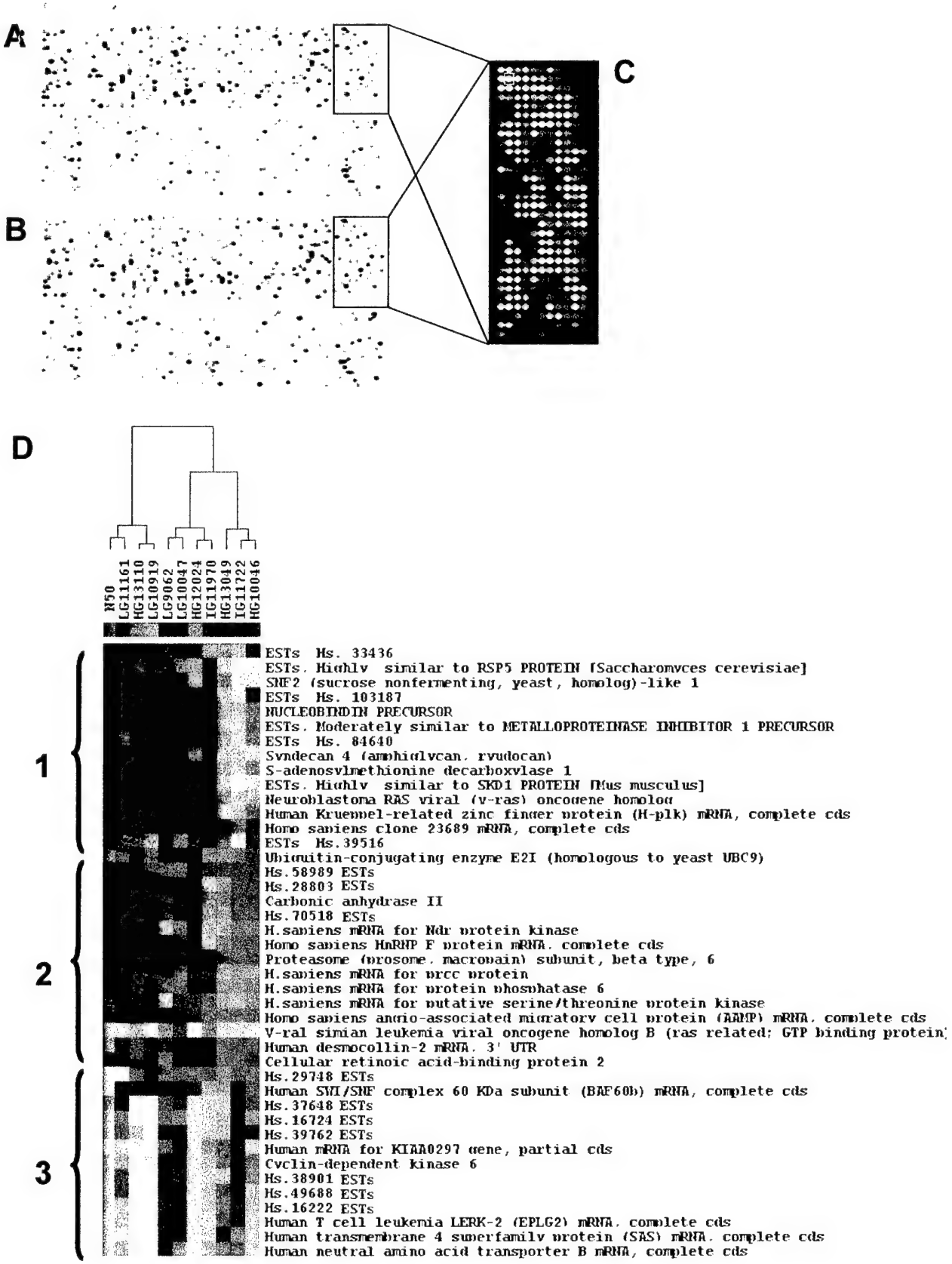
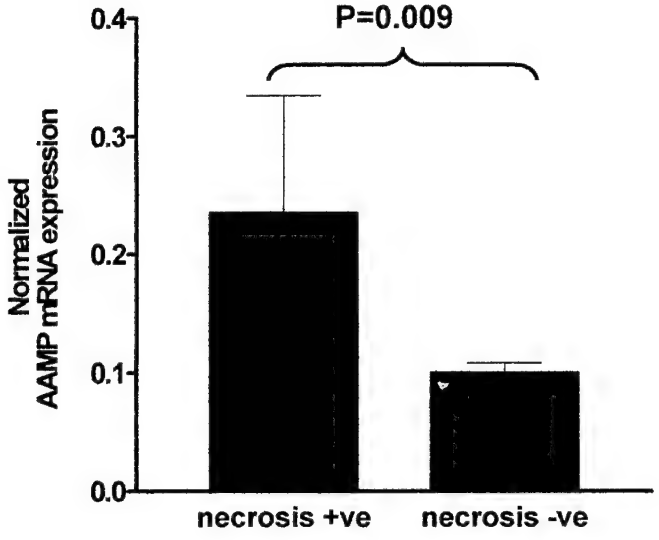

A
B
C
D
M50
LG11161
HG13110
LG10919
LG9062
LG10047
HG12024
IG11970
HG13049
IG11722
HG10046
1
2
3
ESTs Hs. 33436
ESTs. Highly similar to RSP5 PROTEIN [Saccharomyces cerevisiae]
SNF2 (sucrose nonfermenting, yeast, homolog)-like 1
ESTs Hs. 103187
NUCLEOBINDIN PRECURSOR
ESTs. Moderately similar to METALLOPROTEINASE INHIBITOR 1 PRECURSOR
ESTs Hs. 84640
Syndecan 4 (amphiglycan, ryudocan)
S-adenosylmethionine decarboxylase 1
ESTs. Highly similar to SKD1 PROTEIN [Mus musculus]
Neuroblastoma RAS viral (v-ras) oncogene homolog
Human Kruenpel-related zinc finger protein (H-plk) mRNA, complete cds
Homo sapiens clone 23689 mRNA, complete cds
ESTs Hs.39516
Ubiquitin-conjugating enzyme E2I (homologous to yeast UBC9)
Hs.58989 ESTs
Hs.28803 ESTs
Carbonic anhydrase II
Hs.70518 ESTs
H.sapiens mRNA for Ndr protein kinase
Homo sapiens HnRNP F protein mRNA, complete cds
Proteasome (prosome, macropain) subunit, beta type, 6
H.sapiens mRNA for prcc protein
H.sapiens mRNA for protein phosphatase 6
H.sapiens mRNA for putative serine/threonine protein kinase
Homo sapiens amio-associated migratory cell protein (AAMP) mRNA, complete cds
V-ral simian leukemia viral oncogene homolog B (ras related; GTP binding protein)
Human desmocollin-2 mRNA, 3' UTR
Cellular retinoic acid-binding protein 2
Hs.29748 ESTs
Human SWI/SNF complex 60 KDa subunit (BAF60b) mRNA, complete cds
Hs.37648 ESTs
Hs.16724 ESTs
Hs.39762 ESTs
Human mRNA for KIAA0297 gene, partial cds
Cyclin-dependent kinase 6
Hs.38901 ESTs
Hs.49688 ESTs
Hs.16222 ESTs
Human T cell leukemia LERK-2 (EPLG2) mRNA, complete cds
Human transmembrane 4 superfamily protein (SAS) mRNA, complete cds
Human neutral amino acid transporter B mRNA, complete cds

Figure 2

A


P=0.009
0.4
0.3
0.2
0.1
0.0
Normalized AAMP mRNA expression
necrosis +ve
necrosis -ve

B

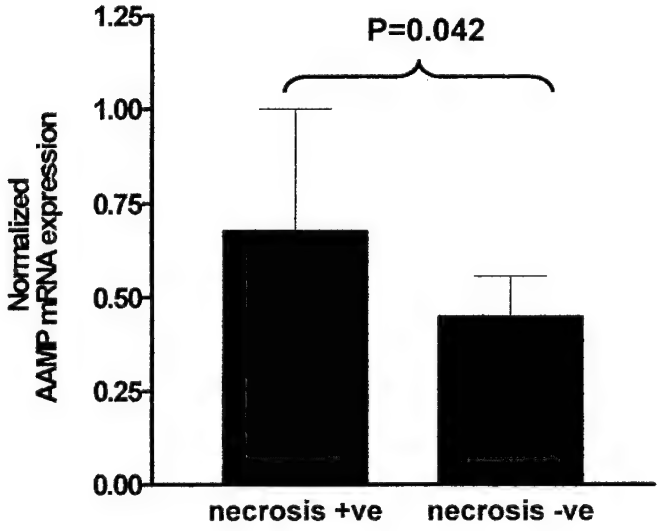

P=0.042
1.25
1.00
0.75
0.50
0.25
0.00
Normalized AAMP mRNA expression
necrosis +ve
necrosis -ve

Figure 3

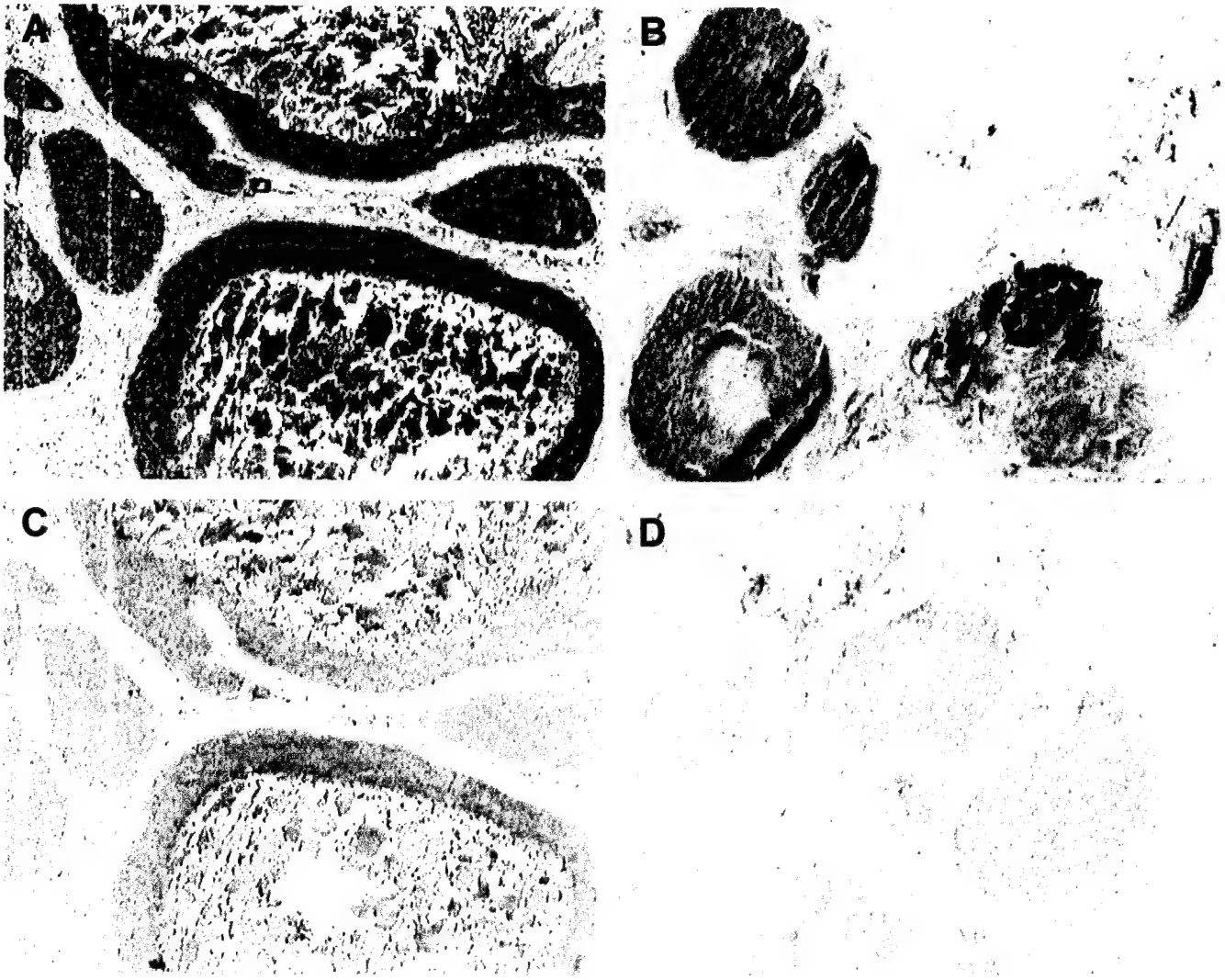


Figure 4

scalpels and the resulting suspension was filtered and stained with 4', 6-diamidino-2-phenylindole (DAPI) dihyrochloride (3μg/ml in 1% sodium citrate and 0.1% NP 40 at pH 6.0). Propidium iodide (PI) stained nuclei analyzed on a Coulter XL cytometer were used for comparison and trout red blood cells were used as internal standards. DAPI stained samples analyzed on the NASA/ACS flow cytometer in general had 2 to 3 fold better resolution (lower coefficient of variation) than the PI stained samples. Thus one could identify cells with DNA index of as low as 1.02 and discriminate between the peripheral blood lymphocytes of male and female subjects on the basis of their DNA content. In contrast to the tight ENV distribution of nuclei from normal tissues, most of the tumor nuclei had enhanced and broad ENV distribution, which made it possible to differentiate between the diploid and the near-diploid (hypo or hyper) tumor cells. In several tumor specimens with broad G0/G1 DNA peaks (PI-stained and analyzed in a conventional cytometer), the dual parametric analysis of ENV and DAPI stained DNA revealed the presence of distinct sub-populations. This dual parametric method based on the simultaneous analysis of DNA and ENV could detect small populations (apparently tumor cells) in secondary sites of metastatic tumors (e.g. lymph nodes) and in surgical resection margins. Supported by the American Cancer Society, Florida Div. Grant (M91-00440) and the National Aeronautics and Space Administration contract (NAS 9-18379).

#307 Layered Expression Scanning - A New Method for High-Throughput Molecular Profiling. Vladimir Knezevic, Galina Baibakov, and Michael R. Emmert-Buck. *20/20 Gene Systems, Inc., Gaithersburg, MD, and National Cancer Institute, Gaithersburg, MD.*

We have designed a new technique for global expression and proteomic profiling of biological samples called layered expression scanning. The method combines complex biological samples (tissue section, cell lysates, gel separated proteins) with a high-throughput array approach to provide a simple and rapid method for comprehensive molecular analysis. The sample of interest is placed adjacent to a set of capture layers, each containing an individual hybridization molecule (antibody or DNA sequence). The specimen is transferred through the membranes and, importantly, the overall two-dimensional architecture and histological relationships within the sample are maintained. As the proteins and nucleic acids are transferred, each target molecule specifically hybridizes to the membrane containing its antibody or complementary DNA sequence. After hybridization each of the membranes are analyzed, thus providing a measurement of the level of expression of each target molecule. As layered expression technology progresses, we envision a laboratory method that will have multiple applications for high-throughput molecular profiling of tissue and cell samples.

#308 Development of a Novel Two-Step Method of Standardized RT-PCR Compatible with High Throughput Gene Expression Analysis of Limited Amounts of cDNA. Erin L. Crawford, Sadik A. Khuder, Robert J. Zahorchak, and James C. Willey. *Gene Express National Enterprises, Inc., Huntsville, AL, and Medical College of Ohio, Toledo, OH.*

Standardized RT-PCR (StaRT-PCR) (Gene Express National Enterprises, Inc., Huntsville, AL) is a powerful tool that allows for the direct comparison of numerical gene expression values between samples and laboratories. In this study, a novel two-step method of StaRT-PCR was developed that allows for the measurement of a substantially greater number of gene expression values without using increased amounts of cDNA and without compromising ability to detect rare transcripts in a statistically significant manner. In addition, the feasibility of comparing StaRT-PCR products from the same cDNA detected using three methods; agarose gel electrophoresis, capillary electrophoresis (CE) (ABI Prism 310 Genetic Analyzer) and microfluidic based CE using the Agilent 2100 Bioanalyzer, was determined. Two-step StaRT-PCR was conducted using two rounds of amplification. In step one, cDNA, competitive template (CT) mix and primer pairs for 9 genes were combined with buffer and enzyme and amplified 5, 8, 10 or 35 cycles. In step two, PCR products from step one were diluted and aliquots of the dilutions were placed in new reaction tubes with buffer, enzyme and a primer pair specific for 1 of the 9 genes and amplified an additional 35 cycles. No additional cDNA or CT mix was added to this second reaction. PCR products from step one could be diluted as much as 100,000-fold and still be quantified following amplification in step two. In contrast, a 100,000-fold dilution of the cDNA and CT mix used in step one followed by one round of 35 cycles with one primer pair did not yield any detectable product. Thus, using two rounds of amplification, the same amounts of cDNA and CT mix that typically are used to obtain 1 gene expression measurement when only one round of amplification is used can be used to obtain 100,000 gene expression measurements without loss of sensitivity to detect rare transcripts. No significant differences between gene expression values obtained by this method and values obtained by control reactions were detected. StaRT-PCR gene expression values detected by the three electrophoresis methods were statistically the same for 14/15 genes evaluated. Values obtained for one gene, E2F5, varied significantly depending on the method of detection, possibly due to measurement of non-specific products produced by the primer pair for this gene. With primer optimization, it is likely that StaRT-PCR gene expression values obtained by any of the three methods of detection can be directly compared and placed in a common database. J.C.W. and R.J.Z. have a significant financial interest in Gene Express National Enterprises, Inc., which produces StaRT-PCR reagents and protocols.

#309 cDNA Microarray Detection by Enzyme Colorimetric Method. Yuh-Pyng Sher, Yueh-Jung Lee, and Konan Peck. *Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan.*

The cDNA microarray provides efficient tool in quantifying large number of gene expression simultaneously. Basic concept is depositing hundreds or thousands of target samples, usually PCR products from cDNA library, on solid substrates (glass slide or filter membrane) and hybridizing with labeled complex probes prepared from cell or tissue RNA extracts to detect expression signal. Enzyme colorimetric detection method is alternatives to fluorescence detection in quantify gene expression patterns on filter membrane that is more accessible to academic laboratories. The method requires 2-5 μ−gram of mRNA labeled with biotin-16-dUTP and/or digoxigenin-11-dUTP for a hybridization reaction to a 9600-feature array. Due to large amount of RNA required per hybridization, most of the applications of cDNA microarray are currently limited to RNA derived from cultured cells. For enzyme colorimetric detection, and laser-induced fluorescence as well, a signal amplification method based on the modified catalyzed reporter deposition (CARD) method can be applied to improve the detection sensitivity. Using CARD amplification, we improve the detection limit by 32-64 fold better than the regular colorimetric method. In addition to signal amplification, sample amplification methods that enrich the amount of RNA molecules for hybridization have been tested in several research labs. These sample amplification methods can be coupled with any of the detection methods to improve the detection limit to the level allowing the use of μ−dissected tissue specimens. In this work we describe a sensitivity enhancement method, which combines one round of sample amplification with signal amplification in cDNA microarray system and improve the detection limit to about 10000 folds compared with the regular colorimetric method. Besides, we développed a new approach, semi-CARD to quantify the amount of target material present in each spot and gene expression pattern in one hybridization experiment to normalize the variation of the spotting material on different membranes.

#310 Differential Gene Expression Analysis of Microdissected Ductal Carcinoma in Situ (DCIS) of the Breast. Adewale Adeyinka, Ethan D. Emberley, Charles C. Wykoff, Adrian L. Harris, Leigh C. Murphy, and Peter H. Watson. *John Radcliffe Hospital, Oxford, UK, and University of Manitoba, Winnipeg, MB, Canada.*

In situ breast carcinomas are now recognized as a spectrum of diseases with different morphology, biological and clinical behavior. We hypothesize a molecular/genetic basis for these differences and have, therefore, compared the gene expression profile, using cDNA microarray membranes, of 4 high-grade and 6 non-high-grade (Van Nuys classification system) microdissected ductal carcinoma in situ of the breast. Tumors for analysis were obtained from the NCIC Manitoba breast tumor bank. ^{33}P-labaled total RNA from tumors were hybridized to GF200 Human Gene Filters (Research Genetics) containing 5,184 spotted cDNAs. Analysis of our data, employing the Pathways 2.01analysis software (Research Genetics), showed that an average of 61 (S.D. = 40) cDNAs were over expressed (greater than or equal to 1.8 fold difference in expression) in high-grade tumors compared with non-high-grade tumors. Amongst these sequences, at least 33 cDNAs were found to be over expressed in three or more pairs of high-grade vs low grade tumors and a small subset of these sequences were also found to be overexpressed in breast cell lines subjected to hypoxia. These findings show that there are consistent differences in the gene expression pattern of high-grade and nor-high-grade in situ breast tumors. Some of these differentially expressed genes may play an important role in determining the biologic and clinical behavior of these tumors and further study is warranted to confirm the differential expression of these genes at the protein level and to determine their relevance in breast cancer progression.

#311 Protein Expression Profile of Lung Tumor and Normal Lung Using Surface Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry: Potential for Molecular Characterization and Biomarker Discovery. Ashley S. Nix, Martin D. Snipes, Michael J. Campa, and Edward F. Patz, Jr.. *Duke University Medical Center, Durham, NC.*

Elucidating the differences in protein expression between tumor and normal tissue would provide more insight into malignancies and potentially lead to improvements in cancer diagnostics and therapy. These data could also be used for phenotypic characterization of tumors and biomarker discovery. In this investigation we used surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI) to analyze protein expression patterns in specimens of lung tumors and compared this to normal lung tissue. SELDI, a newly described tool for proteome analysis, is similar in principle to MALDI-TOF but has the added advantage of offering several different surface chemistries for sample application. By analyzing samples using multiple surface chemistries, it is possible to survey the expression patterns of several hundred different proteins. This feature also permits partial purification of proteins on the sample application surface. In the present study, we analyzed each specimen using 4 different surfaces to capture distinct classes of proteins: anionic, cationic, hydrophobic, and those with metal binding sites. Specific questions addressed include sample preparation, albumin interference, and reproducibility. We show that SELDI, with its combination of rapid data acquisition, small sample size, and versatility, is ideally suited for proteome analysis.

CURRICULUM VITAE

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1985 -1987 Medical Officer, Garki, Abuja, Nigeria.
1991 - 1994 Resident, Department of Pathology, University of Ilorin Teaching Hospital, Ilorin, Nigeria.

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1987 -1991 Lecturer II, Department Of Human Anatomy, Faculty of Medicine, Ahmadu Bello University, Zaria, Nigeria.
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Awards and Grants

1993 Pathology Award of the Nigerian National Postgraduate Medical College, Nigeria.
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1997 Research grant, Swedish Society for Medical Research, Stockholm, Sweden.
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2000-2001 Postdoctoral Fellowship grant, The Susan G. Komen Breast Cancer Foundation, Inc. Dallas, Texas, U.S.A.
2001 Postdoctoral Traineeship Award, Breast Cancer research Program. United States Army Medical Research and Materiel Command.

Membership of Professional and academic bodies

(1) The Nigeria Medical Association
(2) American Association for Cancer Research

Participation at, courses and workshops

(1) UNESCO sponsored workshop on genetic engineering. Enugu, Nigeria. 1988
(2) Advanced molecular cytogenetics course, Cold Spring Harbor Laboratory, New York, U.S.A., March, 1998.

Scientific Publications:

18 research articles.
11 abstracts of presentations at International scientific meetings.

Publications

1. **Adeyinka A** (1989): Karyotype analysis in patient management. Nig Med Practitioner 18: 43-48.

2. Ettarh RR, **Adeyinka A** (1989): Bronchial angulation; a radiological study. In Vaidya MC (ed): Recent Advances in Anatomy. India: Macmillan, pp. 209-210.

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2. **Adeyinka A**, Oladimeji SB, Evaluation of chromosome aberrations in individuas exposed to formalin: report of a pilot study. Presented toThe VIth international congress of the Anatomical Society of West Africa. Ile-Ife, Nigeria. 1991.

3. **Adeyinka A**, Pandis N, Nilsson J, Idvall I, Mertens F, Petersson C, Heim S, Mitelman F, Different cytogenetic patterns in skeletal breast cancer metastases. Presented to the 5th European workshop on cytogenetics and molecular genetics of human solid tumours. Baveno Stresa, Italy. 1996.

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5. **Adeyinka A**, Mertens F, Bondeson L, Garne JP, Borg Å, Baldetorp B, Pandis N. Cytogenetics of male breast cancer progression. Presented to the 2nd Meeting on cancer genetics and tumor suppressor genes, Cold Spring Harbor Laboratory, New York, U.S.A., August, 1998.

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